Ultrastructural study of hepatic stellate cells (Ito cells) after teratogenic drug treatment in pregnancy: Experimental study in Balb/c mice.

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ABSTRACT: *Introduction-Aim of the study:* Ito cells are the hepatic stellate cells in the space of Disse. They play a key role in the development and regeneration of liver. Retinoid analogues are used therapeutically, while hydroxyurea (HU) is an antiretroviral and chemotherapeutic agent. The aim of this study is to evaluate the effect of the above substances on Ito cells' ultrastructural morphology by TEM observations.

Materials-Methods: Six groups of pregnant Balb/C mice were used for the study. All animals were treated on gestational days 10th, 11th, 12th. The first group was treated with All-Trans Retinoic Acid (RA), the second group with Retinyl Palmitate (RP), the third group with a combination of 13-cis RA and RP and the fourth group with HU. The fifth and the sixth groups were the control animals. Animals were sacrificed on the 19th gestational day. Livers from all animals were removed and were properly prepared for TEM observation.

Results: Observation of the liver of All-Trans RA treated pregnant animals revealed Ito cell activation. Microbodies similar to peroxisomes and lysosomes were present in their cytoplasm. Similar findings were found in RP treated animals. In the activated stellate cells, the presence of microfibrils around their nuclei appeared with chromatin condensation in the periphery. In the liver of animals treated with a combination of RP and 13-Cis RA, microfibrils in the cytoplasm, fibrosis and extracellular edema were observed around the sinusoids. In the liver of HU treated animals, peroxisomes with different density were observed in the hepatocyte cytoplasm, surrounded by lipid droplets. In the cytoplasm of activated Ito cells, low density peroxisomes were observed. Fibrosis and extracellular edema were observed surrounding the sinusoids among the stellate cells and in the space of Disse.

Conclusions: This ultrastructural study indicates that the drugs induced Ito cell activation and caused possibly irreversible damage to liver parenchyma.

Key Words: Ito cells, Ultrastructural morphology, Liver, Teratogenic drugs, Retinoids, hydroxyurea.

INTRODUCTION

Ito cells belong to the non-parenchymal liver cells and are located in the perisinusoidal space of Disse. In 1876 Carl von Kupffer was the first who described a type of hepatic stellate cells, located in this space. Based on his observations and studies, Kupffer described them as phagocytic cells[1]. Several decades later, in 1951, the Japanese Professor of Anatomy Toshio Ito observed lipid droplets in the cytoplasm of these hepatic stellate cells and described them as fat storing cells. Since then, these cells were named after him and are nowadays known as Ito cells. However, there are a lot of other names which are also used when referring to Ito cell, such as pericytes, perisinusoidal cells, lipocytes, fat storing cells and of course hepatic stellate cells[2].

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Table 1. Animal groups, number of animals and drugs

Animal Groups	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Number of Pregnant Animal	5	5	5	5	5	5
Drug	All-Trans R.A.	R.P.	13-cis R.A. and R.P.	HU	Corn Oil-(Control)	NaCl 9‰ (Control)
Dosage level	50 mg/ k.b.w	30 mg/ k.b.w	50+30 mg/k.b.w	4,50 mg/ k.b.w	1 ml	1 ml
Gestational days of treatment	10^{th}, 11^{th} and 12^{th}	10^{th}, 11^{th} and 12^{th}	10^{th}, 11^{th} and 12^{th}	10^{th}, 11^{th} and 12^{th}	10^{th}, 11^{th} and 12^{th}	10^{th}, 11^{th} and 12^{th}

All-Trans R.A.: All-Trans Retinoic Acid
R.P.: Retinyl Palmitate
13-cis R.A. and R.P.: 13-cis Retinoic Acid and Retinyl Palmitate
HU: Hydroxyurea

There is a controversial point of view for Ito cell origin. However recently, Baba et al through their research studies demonstrated that Ito cells originate from mesenchyme[3].

Ito cells are known to have at least four distinct functions. They store and release retinoids, they are involved in the construction and breakdown of extracellular matrix, they regulate blood flow in the sinusoids and they produce mediators and cytokines[4]. Hepatic stellate cells play a key role in hepatocellular function by interfering in the development and the regeneration of the liver[5] through extracellular matrix component synthesis, not only under normal conditions, but in liver fibrosis as well[6,7]. Under pathological liver conditions or after liver injury of any cause, Ito cells are transformed from a quiescent state into activated cells which produce extracellular matrix proteins, such as collagen, glycoprotein and proteoglycan[8].

AIM OF THE STUDY

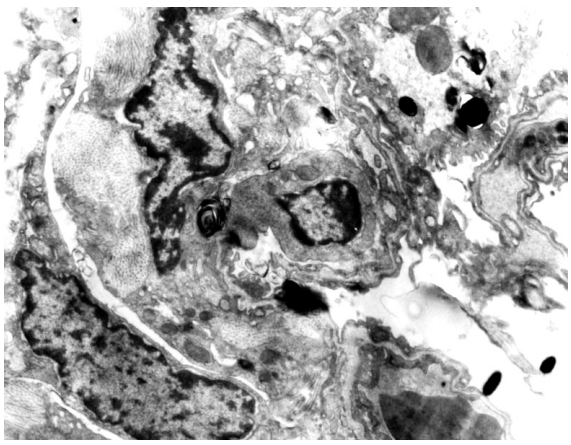
The retinoid analogues 13-cis Retinoic Acid (13-cis R.A.), All-Trans Retinoic Acid (All-Trans R.A.) and Retinyl Palmitate (R.P.), are used therapeutically for several mucosal and skin diseases. Additionally, All-Trans Retinoic Acid is used as a chemotherapeutic agent in acute promyelocytic leukemia. Hydroxyurea (HU) is used in antiretroviral therapy and as a chemotherapeutic drug. These drugs are considered to be teratogenic and toxic agents. On the other hand, it has been described that Ito cells are activated under pathological conditions, such as toxicity of drugs. In the present study we observed the effect of the above substances on the ultrastructural morphology of liver stellate cells, when administrated during early embryogenesis- organogenesis of mice.

MATERIALS AND METHODS

For this study, we treated six (6) groups of pregnant Balb/C mice approximately 30gr, on gestational days 10^{th}, 11^{th} and 12^{th} with: Group 1) All-Trans Retinoic Acid 50 mg/k.b.w., Group 2) Retinyl Palmitate 30 mg/ k.b.w., Group 3) 13-cis Retinoic Acid 50 mg/k.b.w. combined with Retinyl Palmitate 30 mg/k.b.w., Group 4) Hydroxyurea, HU 4,50 mg/k.b.w., Group 5) Corn oil, 1 ml and Group 6) NaCl 9‰ 1 ml. The last two groups were the control animal groups. The pregnant animals were sacrificed on the 19^{th} gestational day.

Livers from pregnant animals were removed and tissue biopsies were taken from the center of the right liver lobe. The samples were cut in small tissue items (1 mm^3), they were immediately immersed and fixed with glutaraldehyde 3% for two hours in 4 °C, post-fixed for one hour in 2% osmium tetroxide (OsO_4) and "in tissue" stained with uranyl acetate 1% and lead citrate. Tissue samples were dehydrated in a degraded series of ethanol solutions and they were finally embedded in Epon resin fixative. Semithin tissue sections 0,5 μm thick were cut and were stained with toluidine blue 2% and intralobular spaces were located under light microscope. Ultrathin sections 600-700 Å were

Group 5. Corn oil treated control.



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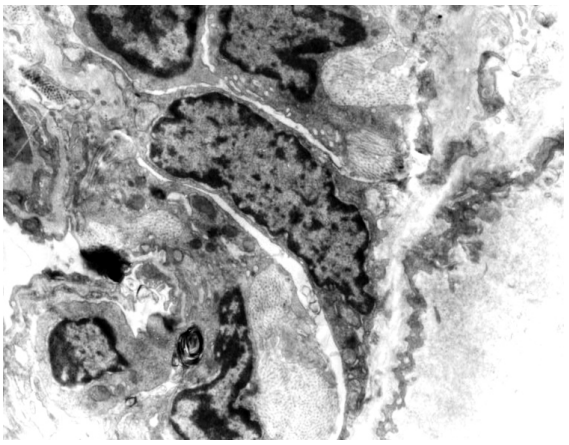

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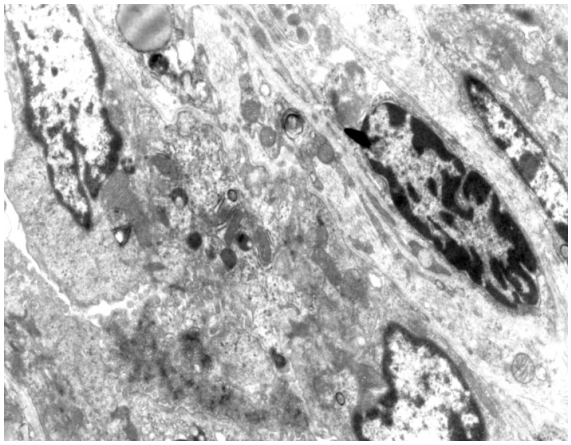
Figures 1 and 2. Ultrathin sections of liver tissue from Group 5 (Corn oil control treated animals). Normal morphology of Ito perisinusoidal cells and lipid concentration as expected. Stellate cells located in the periphery of sinusoidal capillary vessels with lipid droplets located inside their cytoplasm. Magnification x 5.000.

Group 6. NaCl 9‰ treated control.



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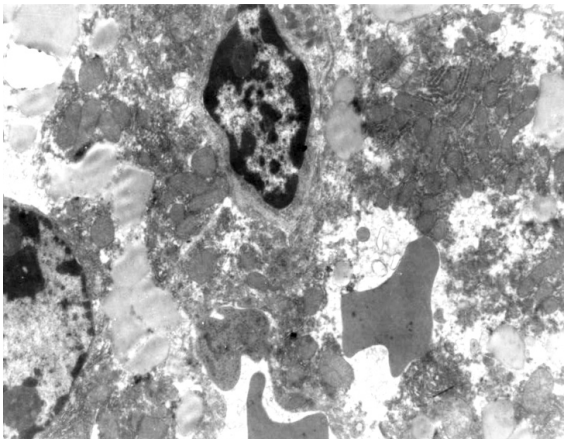

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Figures 3 and 4. Ultrathin sections of liver tissue from Group 6 (NaCl 9‰ control treated animals). Normal morphology of Ito perisinusoidal cells and lipid concentration as expected. Stellate cells located in the periphery of sinusoidal capillary vessels with lipid droplets located inside their cytoplasm. Magnification x 5.000.

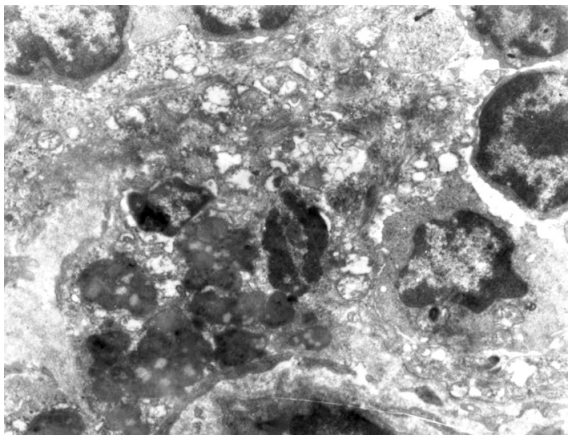
obtained with an ultramicrotome (Leica EM UC6), they were post-stained with lead citrate and observed with the use of Transmission Electron Microscope (Jeol 2000 FX, Tokyo, Japan). Photomicrographs from all liver tissue samples were observed in several magnifications.

RESULTS

Ultrastructural observation of the liver from the control animals (Groups 5 and 6), revealed the expected morphology of Ito perisinusoidal cells and the expected lipid concentration. Stellate cells were located in the periphery of sinusoidal capillary vessels and the lipid droplets were located inside their cytoplasm (Figures 1, 2, 3, 4). The surrounding capillaries appeared to have a normal endothelium and the liver parenchyma did not have any noticeable edema or fibrosis.

Observation of the liver of All-Trans Retinoic Acid treated pregnant animals (Group 1), revealed the activation of Ito perisinusoidal cells, since lipid droplets

Group 1. All-Trans R.A. treated.



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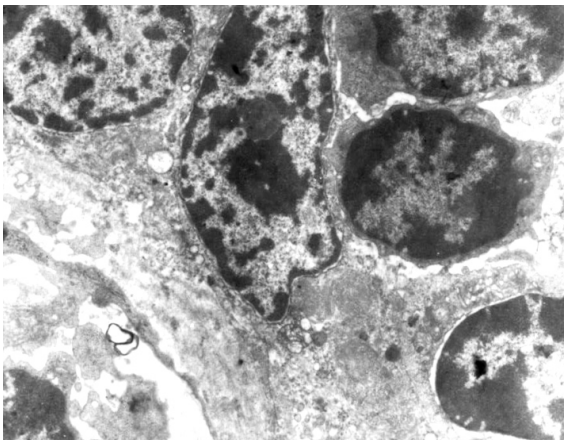

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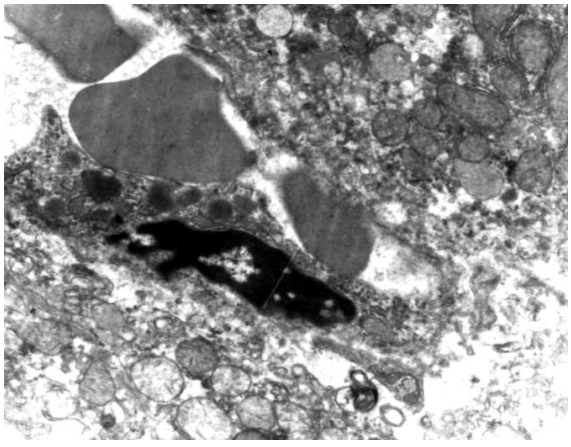

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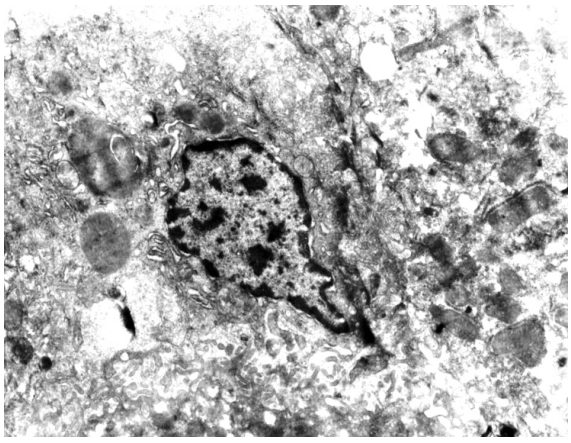
Figure 5. Ultrathin section of liver tissue from Group 1 (All-Trans R.A. treated animals). Activation of Ito perisinusoidal cells without lipid droplets inside their cytoplasm. Microbodies containing fine granular matrix similar to peroxisomes and lysosomes were observed inside Ito perisinusoidal cell cytoplasm.
Magnification x 5.000.

Figure 6. Ultrathin section of liver tissue from Group 1 (All-Trans R.A. treated animals). Lysosomes were observed inside Ito perisinusoidal cell cytoplasm. Magnification x 6.000.

Figure 7. Ultrathin section of liver tissue from Group 1 (All-Trans R.A. treated animals). Lysosomes were observed inside Ito perisinusoidal cell cytoplasm. Magnification x 8.000.

were not observed inside their cytoplasm. Moreover, microbodies containing fine granular matrix similar to peroxisomes (Figure 5) and lysosomes (Figures 5, 6 and 7) were present in their cytoplasm. Similar findings were found in Retinyl Palmitate treated animals (Group 2). An important finding in the activated stellate cells was the presence of microfibrils around their nuclei, which appeared with chromatin condensation in the periphery (Figures 8 and 9). In the liver of animals treated with a combination of Retinyl-Palmitate and 13-Cis retinoic Acid (Group 3), apart from the microfibrils in the cytoplasm, fibrosis and extracellular edema were observed around the sinusoids (Figures 10 and 11). In the liver of Hydroxyurea treated animals (Group 4), more striking findings were observed. Peroxisomes with different density were observed in the hepatocyte cytoplasm, surrounded by lipid droplets. In the cytoplasm of activated Ito cells low density peroxisomes were observed (Figure 12). Fibrosis and extracellular edema were observed surrounding the

Group 2. Retinyl Palmitate treated.



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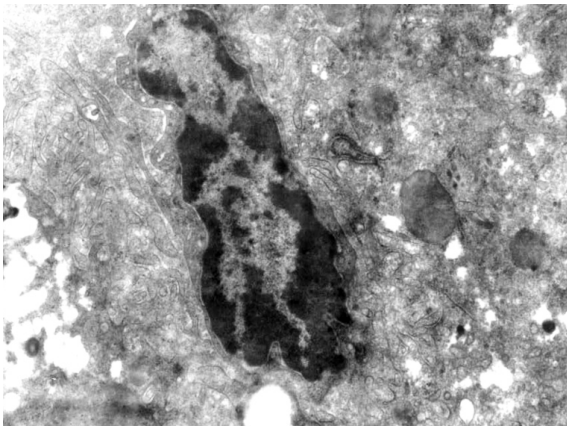

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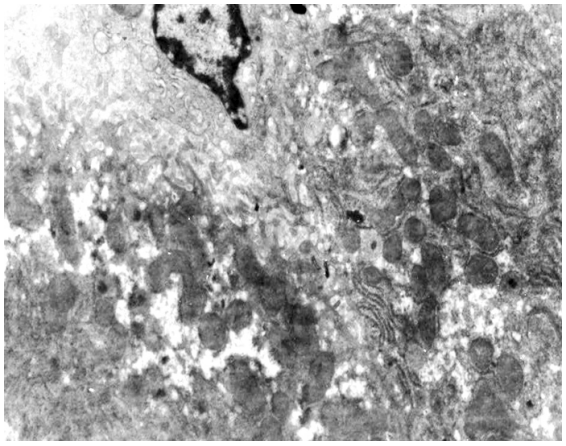
Figures 8 and 9. Ultrathin sections of liver tissue from Group 2 (Retinyl Palmitate treated animals). Activated stellate cells with microfibrils around their nuclei. Magnifications x 5.000 (8), x 8.000 (9).

Group 3. Retinyl-Palmitate and 13-Cis Retinoic Acid treated.



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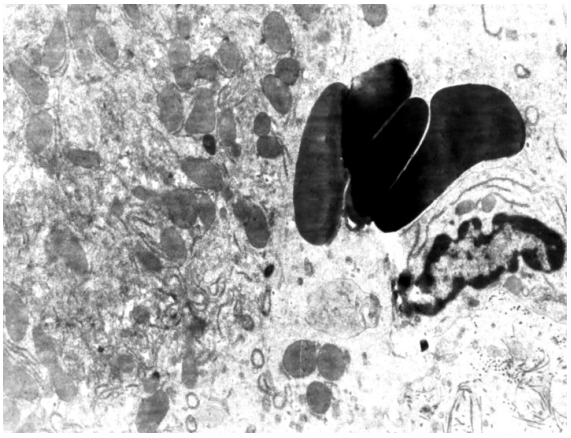

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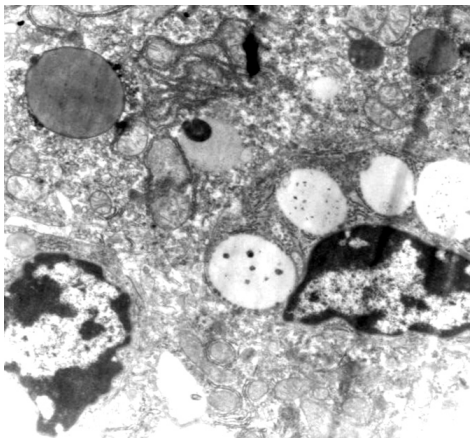
Figures 10 and 11. Ultrathin sections of liver tissue from Group 3 (combination of Retinyl-Palmitate and 13-Cis Retinoic Acid treated animals). Fibrosis and extracellular edema were observed around the sinusoids. Microfibrils were observed in stellate cell cytoplasm.Magnification x 5.000.

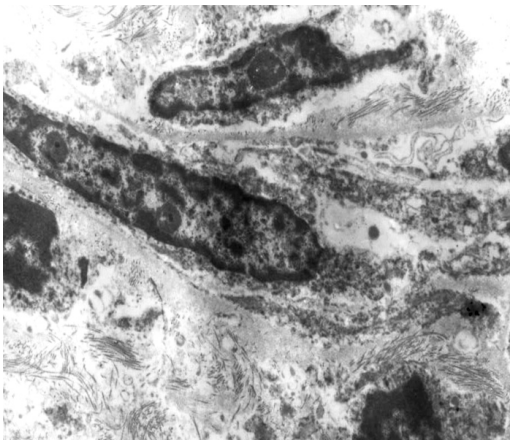
sinusoids among the stellate cells and in the space of Disse (Figures 13, 14 and 15).

DISCUSSION

It has been described that up to 80% of the human body's vitamin A is stored in the liver. Under normal conditions Ito cells are the main site of vitamin A storage. This is stored in the form of retinyl palmitate in cytoplasmic lipid droplets which exert a major role in the homeostasis of the vitamin. It has been described that Ito cells which are located in periportal regions store much more vitamin A than those lying in pericentral regions[9,10]**.** Vitamin A enters Ito cells as a complex (vitamin A - Retinol Binding Protein) by means of endocytosis[11]. This procedure is mediated through Retinol Binding Protein receptors which are expressed on Ito cell surface[9]**.** Hepatic stellate cells have also been found to contain large amounts of cholesterol, triglycerides and free fatty acids[5,12]. Furthermore, proteins that are involved in retinoid metabolism have

Group 4. Hydroxyurea treated.





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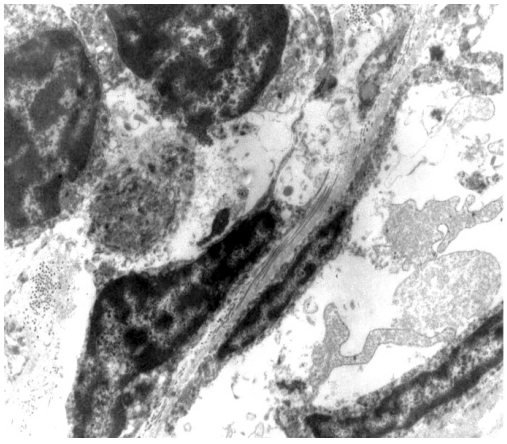

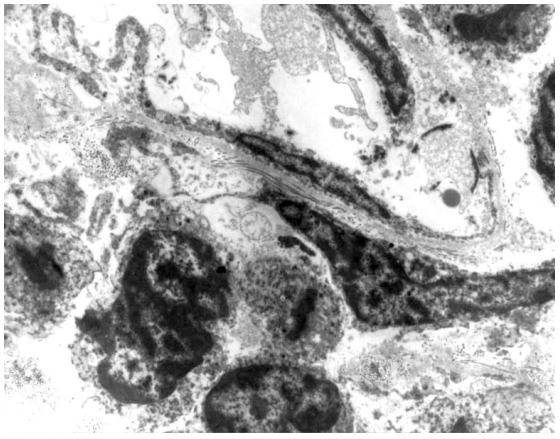

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Figure 12. Ultrathin section of liver tissue from Group 4 (Hydroxyurea treated animals). Peroxisomes with different density were observed in hepatocyte cytoplasm, surrounded by lipid droplets. Activated Ito cells with low density peroxisomes inside their cytoplasm. Magnification x 5.000.

Figures 13 - 15. Ultrathin sections of liver tissue from Group 4 (Hydroxyurea treated animals). Fibrosis and extracellular edema surrounding the sinusoids and among the stellate cells in Disse space. Magnification x 5.000.

also been identified, among which retinol palmitate hydrolase, cellular retinol-binding protein and cellular retinoic acid-binding protein[13,14].

Ito cell activation is a two-stage process including a) initiation, which is also called "proinflammatory" stage and which is the phase of stimulation (with stimuli from neighboring cells and from injured liver parenchyma) and b) perpetuation, which includes a sequence of events as a result of Ito cell response to the stimuli of the first phase. These events of the second stage are described by Friedman and include: proliferation of hepatic stellate cells, cytokine release-chemotaxis-WBC chemoattraction, fibrogenesis, degradation, contractility and retinoid loss[15]. During activation Ito cells proliferate, lose their initial star shape and transform from quiescent and rich in vitamin A cells into deficient in vitamin A proliferative cells. These new cells look like myofibroblasts and are contractile. This process is also known as "transdifferentiation"[16]. Anti-desmin antibody is a marker of quiescent (non activated) stellate cells, while anti-alpha-smooth actin antibody is a marker of activated stellate cells[17]. Activated Ito cells proliferate and synthesize large amounts of extracellular matrix proteins and thus they

are thought to play a major and crucial role in hepatic fibrogenesis[18]. In recent years several papers deal with Ito cells in health and disease. Kazuhiko Besshi et al studied the liver of rats fed with a choline deficient diet, which is known to cause fatty liver and hepatic fibrosis, under light and electron microscopy. This was done in an effort to find out whether it is the reduction of Vitamin A in hepatic stellate cells that induces fibrosis or fibrosis that causes the reduction of vitamin A lipid droplets in Ito cells. The writers were the first to present evidence, with the help of light and electron microscopy, that activation of quiescent Ito cells comes 2 weeks before induction of fibrosis, thus concluding that it is the transformation of Ito cells that causes liver fibrosis[19].

Higashi et al used an electron microscope to study rat livers that had undergone partial hepatectomy and found out that the lipid droplets with vitamin A decreased in number until the third day after hepatectomy and returned to normal levels 2 weeks after the surgical procedure. Their results suggest that Ito cells may change their ability to store Vitamin A during liver regeneration[20]. Another important role of the hepatic stellate cells is that of immunoregulation through their action as intrahepatic antigen presenting cells[21]. Moreover, Ito cells seem to participate and enhance the inflammatory response since they produce chemoattractants for polymorphonuclear and mononuclear leucocytes thus causing their infiltration in the tissues[5]. It is mentioned in the literature that a great variety of other biological agents are also produced and secreted by Ito cells such as lipoproteins, e.g. Apolipoprotein E and prostaglandines[22] including PGE1 and PGE2[23].

A great number of transforming growth factors and cytokines have also been found to be associated with hepatic stellate cells. Indicatively, we mention the epidermal growth factor[24], the platelet derived growth factor[25], fibroblast growth factors a-FGF and b-FGF[26], as well as cytokines such as serotonin[27], endothelin-1[28,29] and thrombin[30].

Several studies have been made in order to clarify the exact role of Vitamin A on hepatic stellate cell activation, which could help us understand better the mechanism of liver fibrosis. Nevertheless, it is still not clear whether vitamin A inhibits or promotes the activation process. Davis et al studied the effects of retinoic acid on hepatic stellate cell lines concerning the cell proliferation and collagen production. They found out that retinoic acid not only had a strongly inhibitory role on cell proliferation, but they also observed a decrease in collagen production. Their findings "in vitro" are indicative of a regulatory role of retinoic acid in hepatic stellate cell activation, and in liver fibrosis[31].

Hellemans et al also studied "in vitro" the effect of All-Trans Retinoic Acid and 9-cis Retinoic Acid on the proliferation of activated hepatic stellate cells and on the synthesis of the components of extracellular matrix. According to their study these two potent Vitamin A derivatives do not exert similar effects on activated hepatic stellate cells i.e. different forms of retinoic acid affect hepatic stellate cells in different ways. More specifically, All-Trans Retinoic Acid was found to induce the reduction of synthesis of extracellular matrix proteins such as fibronectin, laminin and collagen types I and III, without affecting the proliferation of the activated hepatic stellate cells. On the other hand, treatment with 9-cis Retinoic Acid caused a reduction up to 35% in the proliferation of the activated hepatic stellate cells, while only collagen type I synthesis was reduced[32].

Similar research studies on "in vitro" response of activated hepatic stellate cells' after exposure to natural and synthetic retinoic acids concluded that the latter have a different impact on Ito cell proliferation and on collagen and fibronectin production[33].

According to our observations the teratogenic drugs All-Trans Retinoic Acid, Retinyl Palmitate, 13-Cis Retinoic Acid and Hydroxyurea can activate Ito stellate cells and can cause possibly irreversible damage to liver parenchyma.

Υπερμικροσκοπική μορφολογία των αστεροειδών κυττάρων του ήπατος (κύτταρα του Ito) μετά από τη χορήγηση τερατογόνων ουσιών σε κυοφορούντα πειραματόζωα. Πειραματική μελέτη σε Balb/c μύες.

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ΠΕΡΙΛΗΨΗ: *Εισαγωγή-Σκοπός:* Τα κύτταρα του Ito είναι αστεροειδή κύτταρα του ήπατος στο χώρο του Disse. Διαδραματίζουν σημαντικό ρόλο στην ανάπτυξη και την αναγέννηση του ήπατος. Τα Ρετινοϊκά παράγωγα χρησιμοποιούνται στη θεραπεία δερματοπαθειών και στη χημειοθεραπεία της οξείας προμυελοκυτταρικής λευχαιμίας. Η Υδροξυουρία χρησιμοποιείται στην αντιρετροϊική και χημειοθεραπευτική αγωγή. Σκοπός της εργασίας είναι η εκτίμηση της επίδρασης των ουσιών στην υπερμικροσκοπική μορφολογία των κυττάρων του Ito με το Ηλεκτρονικό Μικροσκόπιο (ΗΜ) Διέλευσης.
Υλικά-Μέθοδος: Χρησιμοποιήθηκαν έξι ομάδες κυοφορούντων μυών της φυλής Balb/C. Στη 10η, 11η και 12η ημέρα κύησης, χορηγήθηκαν: στην 1η ομάδα All-Trans Retinoic Acid, στη 2η ομάδα Retinyl Palmitate, στη 3η ομάδα συνδυασμός 13-cis Retinoic Acid και Retinyl Palmitate, ενώ στην 4η ομάδα χορηγήθηκε Hydroxyurea. Στην 5η και 6η ομάδα (ομάδες ελέγχου) χορηγήθηκαν αντίστοιχα αραβοσιτέλαιο και NaCl 9‰. Τα πειραματόζωα θυσιάστηκαν τη 19η ημέρα της κύησης. Το ήπαρ από κάθε πειραματόζωο αφαιρέθηκε και μετά κατάλληλη προετοιμασία παρατηρήθηκε στο ΗΜ Διέλευσης.
Αποτελέσματα: Στην 1η ομάδα παρατηρήθηκε ενεργοποίηση των κυττάρων του Ito. Στο κυτταρόπλασμά τους διαπιστώθηκαν λυσσοσωμάτια και μικροσωμάτια ομοιάζοντα με υπεροξεισωμάτια. Ανάλογα ήταν τα ευρήματα στη 2η ομάδα. Στην 3η ομάδα παρατηρήθηκε επιπλέον ίνωση και εξωκυττάριο οίδημα γύρω από τα κολπώδη τριχοειδή. Στην 4η ομάδα, στο κυτταρόπλασμα των ηπατοκυττάρων παρατηρήθηκαν υπεροξυσωμάτια διαβαθμιζόμενης πυκνότητας, περιβαλλόμενα από λιποσταγονίδια. Τα κύτταρα του Ito ήταν ενεργοποιημένα, ενώ στο κυτταρόπλασμά τους διαπιστώθηκαν υπεροξυσωμάτια χαμηλής πυκνότητας. Τέλος, παρατηρήθηκε ίνωση και εξωκυττάριο οίδημα γύρω από τα κολπώδη τριχοειδή, ανάμεσα στα κύτταρα του Ito και στο χώρο του Disse.
Συμπεράσματα: Η υπερμικροσκοπική μελέτη της μορφολογίας των κυττάρων του Ito, υποδεικνύει ότι οι χορηγηθείσες ουσίες προκάλεσαν την ενεργοποίησή τους και πιθανώς μη αναστρέψιμες βλάβες στο ηπατικό παρέγχυμα.

Λέξεις Κλειδιά: Κύτταρα του Ito, Υπερμικροσκοπική μορφολογία, Ήπαρ, Τερατογόνα φάρμακα, Ρετινοϊκά παράγωγα, Υδροξυουρία.

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